

## Exhibit B

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We have developed a monoclonal antibody (mab M9) against antithrombin that has the same properties as the one against the complexed/cleaved protein C inhibitor. A similar approach was used to obtain this mab (M9) as for the identification of the mab that is specific for the complexed/cleaved form of PCI. Like the antiPCI mab M9 against antithrombin III is specific for the loop inserted forms of the inhibitor, i.e. the complexed/cleaved inhibitor. In antithrombin III a third loop-inserted form, that does not seem to have a counterpart in the protein C inhibitor, has been identified; the so called latent form (see Zhou, A. et al. (1999) Blood 94; 3388-3396 and P. W. Gettins (2002) Chemical Reviews 102; 4751-4803).

Mab M9 binds all three forms of loop-inserted antithrombin III (complexed, cleaved and latent). This is illustrated in Fig 1 that shows a comparison of competitive

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binding curves obtained with our mab and the complexed, cleaved and latent forms of antitrombin as well as with the native uncomplexed form of the inhibitor (i.e. the dominant form of the inhibitor in human plasma). It is obvious that the M9 has more than 1000-fold higher affinity for the three loop-inserted forms of antithrombin than it has for the native form.

Production of a monoclonal antibody that is specific for the three forms of loop-inserted antithrombin is straight forward and has been described for the production of the monoclonal against PCI. The crucial point is the screening of the clones. This requires screening with both the native inhibitor and one of the three cleaved forms, for instance antithrombin in complex with thrombin of factor Xa..

Heparin Sepharose affinity chromatography has been used to separate native and latent antithrombin III (Zhou, A. et al. (1999) Blood 94; 3388-3396). We have also used this approach (Fig 2). The latent form elutes earlier than the native form. This form is recognized by M9 in a sandwich assay where biotinylated M9 is catcher mab and an unrelated mab reporter mab (M38). The later eluting native antithrombin has, in contrast to the latent form, inhibitory activity against thrombin. However, this form is not recognized by mab M9. The protein C inhibitor and antithrombin III are so called serpins, i.e. serine protease inhibitors that work by the same mechanism. These inhibitors also include heparin cofactor II, antitrypsin, antichymotrypsin and numerous other inhibitors. They are all homologues and operate by the same mechanism. We think we have shown that our claim is valid, i.e. that our approach makes it possible to make monoclonal antibodies against the loop-inserted forms of other serine protease inhibitors that belong to the so called serpin family.

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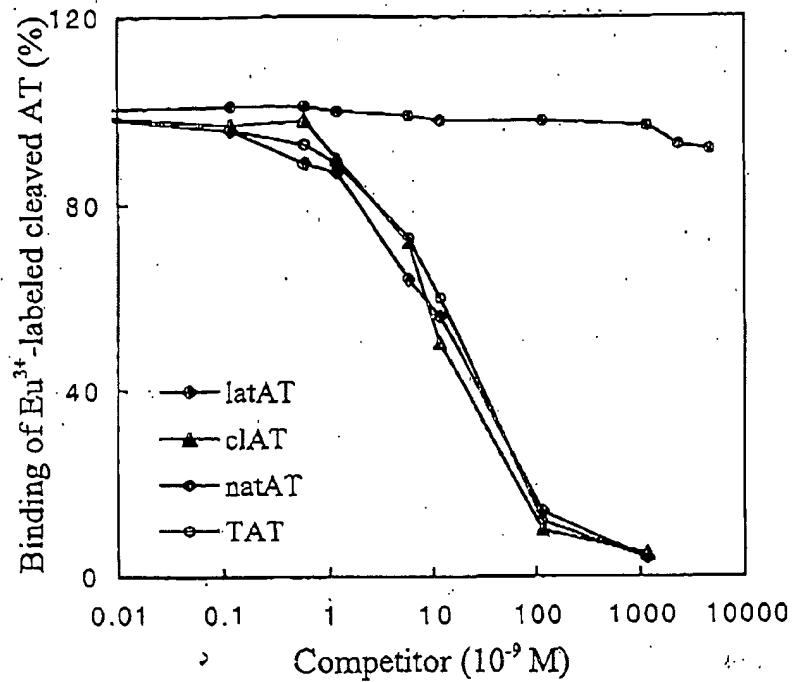
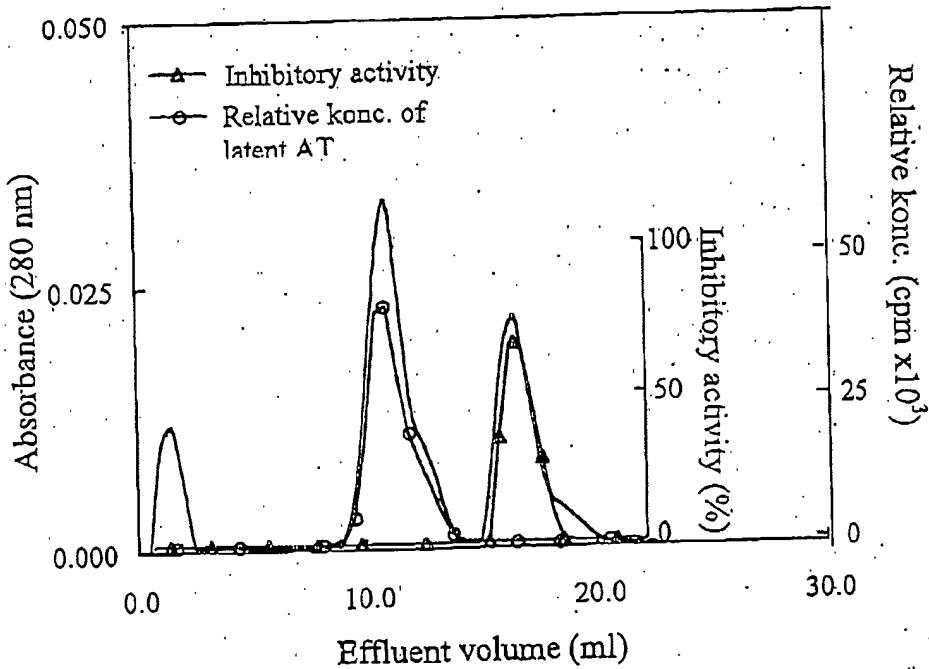


Fig. 1. Competition for mab M9 between Eu<sup>3+</sup>-labelled cleaved antithrombin III (AT) and increasing concentrations of different forms of antithrombin III. Native AT (■), latent AT (◆), cleaved AT (▲) and TAT (○) competed with Eu<sup>3+</sup>-labeled cleaved AT for the binding to M9. Increasing concentrations (0-400 µg/ml) of native, latent cleaved free and thrombin-complexed AT (25 µl) in Assay buffer (Wallac) were added to 25 µl Eu<sup>3+</sup>-labeled cleaved AT (10 ng/ml) in a non-adsorptive microtiterplate. The solutions were supplemented with 50 µl biotinylated M9 (0.2 µg/ml). After 30 min incubation the content of each well was transferred to a streptavidin plate and incubated for 1 h. The plate was washed with Wash solution (Wallac). Enhancement solution (Wallac) was added and the fluorescence from M9-bound Eu<sup>3+</sup>-labeled cleaved AT measured in a DELFIA plate fluorometer (Wallac). The y-axis shows the binding of Eu<sup>3+</sup>-labeled cleaved AT to M9 relative to its binding with no competitor present.



**Affinity chromatography of latent and native antithrombin (AT) on HiTrap Heparin HP Column.**

A mixture of equal amounts of native and latent AT was applied to a HiTrap Heparin HP Column, equilibrated with 50 mM Tris-HCl, 0.15 M NaCl pH 7.5 at a flow rate of 0.5 ml/min. The proteins were eluted with a gradient (0.15-1.5 M NaCl). The eluent was monitored at 280 nm (—). Fractions were analyzed in a DELFIA (○) as follows: Aliquots (50  $\mu$ l) of biotinylated M9 (4  $\mu$ g/ml) were transferred to non-adsorptive microtiter plates (Bibby Sterilin Ltd., Staffs, UK). Fractions (50  $\mu$ l), diluted 1/80 in Assay buffer (Wallac, Upplands Väsby, Sweden), were added and the plate was incubated for 30 min at room temperature. The solution from each well was removed to Streptavidin coated plates (Wallac) and incubated for 1 h followed by washing with Wash solution (Wallac). After incubation for 1 h with 100  $\mu$ l of a Eu<sup>3+</sup>-labeled mab, M38 (0.2  $\mu$ g/ml), directed against another part of AT, the plate was washed. Enhancement solution (200  $\mu$ l) (Wallac) was added and fluorescence measured in a DELFIA plate fluorometer (Wallac).

The eluate was also analysed for the ability to inhibit thrombin and to bind to M9 ( $\Delta$ ). Fractions (100  $\mu$ l), diluted 1/25 in TBS containing 0.1 % BSA (w/v) and 4 U/ml heparin (Lövens Kemiske Fabrik, Ballerup, Denmark), were transferred to microtiter plates (Costar) and supplemented with 100  $\mu$ l thrombin (0.2  $\mu$ g/ml) diluted in the same buffer. After 15 min incubation at room temperature, 50  $\mu$ l thrombin substrate S-2238 (Chromogenix, Gothenburg, Sweden) was added and increasing absorbance at 405 nm was recorded for 10 minutes.